Research Note

The Immunomodulatory Effects of Clonidine, an α-2-Adrenergic Agonist, in Laying Hens

H. W. Cheng[1]

Livestock Behavior Research Unit, USDA-ARS, West Lafayette, IN 47907

ABSTRACT The ability of the sympathetic nervous system to regulate chicken immune function was examined. Clonidine, an α-2 adrenergic receptor agonist, was administrated at 2.5, 5.0, or 10.0 mg/L in the drinking water of White Leghorn hens at 48 wk of age. The hens were randomly housed in conventional cages in pairs. Concentrations of plasma IgG (also named IgY) were detected using chicken IgG ELISA, and the percentage of subpopulations of circulating lymphocytes were analyzed using flow cytometry. Compared with controls, treated hens had higher circulating IgG levels and a greater percentage of circulating B cells (Bu-1$^+$ cells, $P < 0.01$) after 1 wk of treatment. Additionally, the percentages of CD8$^+$ cells were consistently higher ($P = 0.07$) in the treated hens compared with controls, whereas the percentages of CD4$^+$ cells and the ratio of CD4$^+$ cell to CD8$^+$ cell were not affected by the treatment ($P > 0.05$). These effects of clonidine on the IgG concentrations and the proportions of B-cells and CD8$^+$ cells were dose-related. The highest increase was found in the hens treated with 10.0 mg/L of clonidine. These results suggest that the sympathetic nervous system is directly involved in regulating the chicken immune system via regulation of α-2-adrenergic receptor activations.

Key words: clonidine, adrenergic receptor, lymphocyte, chicken

2006 Poultry Science 85:452–456

INTRODUCTION

The sympathetic nervous system plays important roles in regulating a variety of cellular activities in the body to maintain physiological homeostasis, including modulation of immune responses to internal and external stimuli. Dense innervations of sympathetic nerve fibers have been found in the primary (the thymus and bone marrow) and secondary (the spleen and lymph nodes) lymphoid organs (Vizi and Elenkov, 2002; Straub, 2004). Released neurotransmitters from those axon terminals, such as dopamine, epinephrine, and norepinephrine, regulate innate and adaptive immune responses by binding to specific receptors on the surface of immune cells, resulting in immune enhancement or suppression, respectively, based on the characteristics of stimulators and their durations (Elenkov et al., 2000).

The discovery of highly specific adrenoceptor agonists and antagonists has opened a new channel for investigating the role of the sympathetic nervous system in regulating physiological homeostasis including immunity. Clonidine, a selective α-2-adrenergic agonist, acts in both the peripheral and central nervous systems (Gilman, 2001). Based on its pharmacological characteristics, clonidine has been clinically used to treat hypertension and attention-deficit/hyperactivity disorder and is also used as an analgesic. In addition, clonidine is reported to produce a variety of other physiological effects. For example, it suppresses oral stereotypes and vocalizations in chickens (Savory and Kostal, 1994), reduces separation distress in monkeys (Harris and Newman, 1987), and has been effective in treating depression, anxiety, and mania in humans and rodents (Arnsten and Goldman-Rakic, 1985; Kafka and Paul, 1986). Clonidine also reportedly improves immunity and reduces inflammation and infection in humans and rodents by directly regulating the functions of immune cells and organs and by indirectly regulating stress response systems, such as the hypothalamic-pituitary-adrenal axis (Jurankova et al., 1995). Similar effects may also occur in chickens, since the neuroendocrine systems that control stress and immune responses behave similarly in birds and mammals (Marsh and Scanes, 1994; Sharma, 1997). In chickens, as in mammals, clonidine may directly and indirectly affect the immune system and alleviate stress responses via central nervous system and peripheral nervous system pathways. The objective of the present study was to investigate the effects of clonidine on circulating immunocompetent cells and IgG concentrations in laying hens.

MATERIALS AND METHODS

Birds

Experimental White Leghorn chicks (Hy-line W-36) were hatched, vaccinated for Marek's and Newcastle dis-

Received July 21, 2005.
Accepted October 24, 2005.
[1]Corresponding author: hwcheng@purdue.edu

eases, and reared under standard management practices in the grower house at Purdue University Poultry Farm. At 17 wk of age, the pullets were randomly assigned to 2 birds per cage, each provided 645 cm^2, in the laying house. Feed and water were provided for ad libitum consumption. Overhead lights were on daily from 0700 until 1900 h initially and were increased by 15 min/wk until a 16L:8D light cycle period was achieved.

At 48 wk of age, 32 cages (64 hens) were randomly chosen and moved to a separate room from other hens. These cages were randomly assigned to 1 of 4 treatment groups described later. The hens were maintained in this same room during the entire experimental period. Feed and water were provided for ad libitum consumption. Overhead lights were on a 16L:8D light cycle. Chicken care guidelines were in strict accordance with the rules and regulations set by the Federation of Animal Science Societies (Craig et al., 1999), and all procedures were approved by Purdue University Animal Use Committee.

Treatments

Following a 7-d acclimation period, the hens were exposed to 1 of the 4 treatments as follows: control group with regular drinking water and treatment groups with water containing 2.5, 5.0, or 10.0 mg/L of clonidine (Sigma, St. Louis, MO). The drinking water (i.e., pure water or clonidine-mixed water) for the control and treatment groups was kept separately in 10-gal containers, which were placed on a digital scale. Water consumption was recorded daily. Based on the schedule of clonidine treatment used in several previous studies in which clonidine was administered from 5 to 9 d (Knight et al., 2001; Amador et al., 2004), clonidine was given for 7 d in the current study. The schedule reflects clonidine's pharmacological basis. In general, clonidine is well absorbed after oral administration, has a half-life from 6 to 24 h, and takes 3 to 4 d to reach steady-state concentrations in plasma (Lowenthal et al., 1988; Hardman et al., 1996). After 7 d of the treatment, the mixed drinking water was drained out and replaced with tap water.

Blood Sampling

One randomly chosen bird from each cage (n = 8 per treatment group) was bled on d 7 of treatment. A 20-mL blood sample was collected through heart puncture using a 20-gauge needle within 2 min of removing the bird from its cage. The heparinized blood samples were centrifuged at 700 × *g* for 15 min at 4°C. Plasma was used for analysis of IgG (also called IgY), and cells were used for analysis of circulating lymphocyte populations (Eicher et al., 2000).

Flow Cytometry Analysis for Immunocompetent Cells

Flow cytometry analysis of the percentage of lymphocyte populations, including $CD4^+$, $CD8^+$, and B-cells was performed using the methods as described by Li et al. (1999) and Kliger et al. (2000), with slight modification. After removing plasma, buffy coat solution (Life Technology, Inc., Frederick, MD) was added immediately to each sample tube. The mixed samples were centrifuged at 700 × *g* for 40 min at 4°C and the buffy coat layer was collected and transfered into a new tube with filled the medium 1640 (Life Technology, Inc.). Leukocytes contained in the buffy coat medium were separated from any remaining red blood cells using Red Blood Cell Lysing Solution (Life Technology, Inc.) by centrifugation at 700 × *g* for 20 min at 4°C. The leukocytes were washed 2 times in 1640 medium by centrifugation for 10 min at 500 × *g*. The cell pellet was resuspended and washed 2 times in FACS buffer (Hanks' balance salt solution without phenol red containing 0.2% NaN_3 and 3% fetal calf serum; Life Technology, Inc.). The density of cells was counted using a Coulter Z1 Cell Counter (Coulter Inc., Kennesaw, GA) and resuspended in the FACS medium at 1×10^6 cells/mL. Then, 200 uL of the cell suspension of each sample was added into separate tubes for phenotype determination using direct fluorescein isothiocyanate- and phycoerythrin-conjugated antibodies for $CD4^+$ and $CD8^+$ immune cells (Southern Biotechnology, Inc., Birmingham, AL), respectively. Based on previous findings that Bu-1 antigen represents a unique surface marker for all B-cells and their precursors (Houssaint et al., 1989; Tregaskes et al., 1996), anti-Bu-1 antibody (Southern Biotechnology, Inc.) was used for B-cell analysis in the present study. Based on our preliminary study, the cells were incubated for 1 h at 4°C with antibody concentrations as follows: 50 μl of 1:50, 1:100; and 1:100 diluted for $CD4^+$, $CD8^+$, and B-cells, respectively. After washing 3 times with FACS solution by centrifugation and then fixed with 1.0% paraformaldehyde, the percentage of labeled cells was determined using a Coulter XL MCL flow cytometry (Beckman Coulter, Inc., Fullerton, CA) with a 488-nm air-cooled argon laser for excitation, a 525 band pass for fluorescein isothiocyanate labels, and a 575-nm band pass for phycoerythrin detection. All necessary negative controls were included, such as cells only, cells plus secondary antibody, and HB2 (antihuman T cells, as an isotypically matched negative control mouse monoclonal antibody; American Type Culture Collection, Manassas, VA; Eicher et al., 2000; Kliger et al., 2000). The results were analyzed using the system II software (Beckman Coulter, Inc.). As there were few effects of the nonspecific binding of the cell only and cells plus secondary fluorescein-labeled antibody (i.e., without the primary antibody), the data was calculated as that the percentage of fluorescent-positive live cells for the specific marker subtracts the background staining by HB2. The results were reported as the percentage of total live cells.

ELISA Assay to Determine Plasma Concentrations of IgG

Concentrations of blood IgG were measured in duplicate using a chicken IgG ELISA quantitative kit (Bethyl

Table 1. Clonidine-induced alteration in the proportions among circulating lymphocyte populations in laying hens

Treatment[1]	$CD4^+$ cells[2]	$CD8^+$ cells	$CD4^+$:$CD8^+$	Bu-1 cells
	(% positive)			(% positive)
Control	31.8 ± 2.2	11.4 ± 1.4	2.79 ± 0.12	9.1 ± 1.1[a]
2.5 mg/L	31.6 ± 2.2	12.5 ± 0.9	2.52 ± 0.11	10.4 ± 1.0[ab]
5.0 mg/L	32.2 ± 2.8	12.9 ± 1.1	2.50 ± 0.09	13.1 ± 1.1[b]
10.0 mg/L	35.8 ± 2.6	13.8 ± 0.8	2.59 ± 0.13	15.2 ± 1.1[b]

[a,b]Means within a column with no common superscript differ significantly ($P < 0.01$).

[1]At 48 wk of age, chickens were randomly exposed to 1 of the 4 treatments as following: control group with regular drinking water, and treatment group with water containing 2.5 mg, 5.0 mg, and 10.0 mg/L of clonidine. Populations of immune cells were determined using flow cytometric analysis on d 7 of treatment.

[2]Values are presented as mean ± SEM (n = 8).

Labs, Inc., Montgomery, TX) with a modification based on the manufacturer's recommendations and the methods used by Yonash et al. (2002) and Schuijffel et al. (2005). Based on our preliminary tests, a plasma dilution of 1 to 20,000 was used in this study, and the control IgG standard curve was from 0 to 500 ng. Briefly, diluted samples and standard plasma were added separately into 96 well ELISA plates coated with affinity purified goat anti-chicken IgG. After incubation, goat anti-chicken IgG conjugated to peroxidase was added at a dilution of 1:1,200 into each well. Two wells containing no test serum were included on each plate as negative controls. Following incubation and washing, the substrate solution for peroxidase (1:1, TMB peroxide substrate A: peroxidase solution B) was added, then treated with stopping reagent (2*M* H_2SO_4). The enzyme-substrate reaction was read on a Microplate Spec using KC4 software (Bio-Tek Instruments, Inc., Highland Park, Winooski, VT), and the concentrations of plasma IgG were determined when compared with the IgG standard curve on each plate and converted to milligrams per milliliter.

Statistical Analysis

The experimental design was completely randomized with treatment as the main effect. Results were assessed statistically using 1-way ANOVA with the cage as the experimental unit and intertreatment differences were analyzed by Newman-Keuls posthoc test. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

The present study demonstrated that clonidine, an α-2-adrenergic agonist, affects the immune system in chickens. Compared with the controls, the treated chickens had a higher percentage of circulating B-cells and a greater concentration of plasma IgG following the administration of clonidine ($P < 0.01$ and $P < 0.05$, respectively; Table 1 and Figure 1). In addition, the percentages of $CD8^+$ cells were consistently higher ($P = 0.07$) in the treated hens compared with controls, whereas the percentages of $CD4^+$ cells and the ratio of $CD4^+$ cells to $CD8^+$ cells were not affected by the treatment ($P > 0.05$). The results are consistent with previous studies and further suggest that the sympathetic nervous system plays an important role in regulating animal immune responses via regulating α-2-adenergic receptor activations (Elenkov et al., 2000; Kimura et al., 2005).

Various subtypes of adrenergic receptors, including α-2-receptors, have been found on different types of immune cells of chickens, including T and B lymphocytes (Randall et al., 1983; Sanders, 1995). In the current study, it has been shown that the functions of the B-cell system can be stimulated by clonidine, resulting in an increase in plasma concentrations of IgG and the percentage of B-cells (Figure 1 and Table 1). The effects of clonidine on the immune system of chickens could result from its α-2-adrenergic agonist activities. Similar to current findings, previous studies have shown that the function of T and B lymphocytes can be modulated by various adrenergic agonists or antagonists in humans and rodents (Lefkowitz, 1982; Doherty, 1988).

The present study showed that clonidine affects different lymphocyte populations differently and hence may exert different effects on humoral- vs. cell-mediated immune system aspects. The unique effects of clonidine on

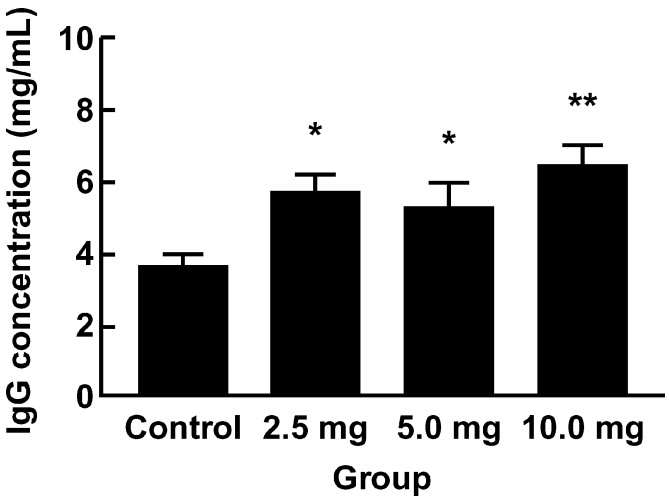

Figure 1. Plasma IgG concentrations in laying hens treated with clonidine. Clonidine, an α-2 adrenergic receptor agonist, was administrated in the drinking water of White Leghorn hens at 48 wk of age. Concentrations of plasma IgG were detected using chicken IgG ELISA on d 7 of treatment. Values are presented as mean ± SEM (n = 8). *$P < 0.05$; **$P < 0.01$.

the different immune systems may indicate that different types and or subtypes of adrenergic receptors were involved in these reactions. Similar to this hypothesis, previous studies have identified that there are unequal distributions of different adrenergic receptors among T and B lymphocytes and their subpopulations. Each type of receptor exhibits unique functions in regulating the production of cytokine, lymphocyte proliferation, antibody secretion, and performing cytotoxic function (Sanders et al., 1997; Anstead et al., 1998). Disruption of the sympathetic nervous system, such as changes of adrenergic receptors and neurotransmitters, affects immunity. Sanders (1995) reported that chemical sympathectomy suppresses cell-mediated responses but enhances antibody responses. The suppression of peripheral blood T lymphocytes but not spleen cells in rats was primarily due to the activation of α-2-adrenergic receptors (Felsner et al., 1995). Although the reason for the different regulation of immune reactions following administration of clonidine remains unclear, it could be the same cellular mechanisms as those reported in humans and rodents because there are similarities between the distribution and function of α-2 adrenergic receptors in birds and mammals (Randall et al., 1983).

In humans and rodents, it has been shown that clonidine has multiple functions in regulating cell- and humoral-immunity, including stimulating the proliferation of splenocytes (Cupic et al., 2001) and increasing lymphocyte blastogenic responses (Jurankova et al., 1995). Clonidine also exhibits antiinflammatory properties by 1) increasing splenocyte interferon-γ production and splenic natural-killer cell activity (West et al., 1999); 2) augmenting circulating leukocytes and neutrophil counts and function (Novak-Jankovic et al., 2000); and 3) boosting macrophage production of tumor necrosis factor-α (Maes et al., 2000) and interleukin-12 (Kang et al., 2003). Clonidine may exhibit immune effects in treated chickens via the similar cellular mechanisms, which are akin to those in mammals.

The characteristic of clonidine-induced immune response in chickens could prove beneficial in chicken management, such as administering clonidine early after birds are relocated from the grower house to layer house for production and during induced molting. These practices are traditionally used in the egg industry and cause birds to be stressed, which increases susceptibility to stress and infections. For example, increases in *Salmonella enteritidis* contamination have been reported during induced molting (Holt, 2003). In agreement with this hypothesis, previous studies have reported that clonidine prevents inflammation, infection, or both in various species of animals, such as increasing resistance to *Mycobacterium avium* in rodents (Weatherby et al., 2003) and modifying lipopolysaccharide responses in chickens (Szelenyi et al., 2000).

In conclusion, the present study demonstrates that clonidine causes an increase in the percentage of circulating of B lymphocytes and IgG concentrations in chickens. Clonidine regulates chicken immune system in a dose-dependent manner by directly acting on α-2-adrenergic receptors located on immune cells.

ACKNOWLEDGMENTS

Thanks to Y. Chen for assistance in conducting the IgG analysis. This work was supported by a USDA-NRI grant.

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